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Newly Synthesized Compound, PABA-Ursodeoxycholic Acid, for Evaluation of Intestinal Bacteria

Makoto TAKAHASHI, Yorinobu MAEDA, Kiyoshi MATSUNO, Hiroyuki TAMURA, Susumu FURUYASHIKI and Mitsuo HARADA

Department of Surgery, Chugoku Rosai Hospital, Kure, Hiroshima 737-01, Japan

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ABSTRACT

From the standpoint of utilizing a distinctive feature of para-aminobenzoic acid (PABA) and intestinal bile acid metabolism, a conjugate of ursodeoxycholic acid with PABA (PABA-UDCA) was newly synthesized for studying whether it can be a good material or not to evaluate enteric bacteria. In incubation experiment, this compound was reasonably deconjugated by cholylglycine hydrolase resulting in the release of PABA. In animal experiment, urinary excretions of PABA were determined during six hours following oral administration of 10 mg PABA-UDCA. Control rats (n=7) excreted 140.1 ± 59.5 μg (mean ± SD) of PABA. By contrast, the rats (n=9) with intestinal antisepsis by antibiotic administration excreted less (18.3 ± 16.7 μg; $P<0.001$) whereas those with intestinal bacterial overgrowth by making enteric blind loop excreted more (451.1 ± 223.6 μg; $P<0.01$). These observations indicate that this new compound is likely to offer a simple and rapid method for evaluation of the intestinal microorganisms.

There have been some methods to evaluate activities of the intestinal bacteria. Among them breath test utilizing ^{14}C-labeled glycocholate has been most well known[3], and was reported to be useful for the diagnosis of the intestinal bacterial overgrowth in such conditions as blind loop syndrome and so on[10]. However this method has flaws of using radioisotope and being troublesome.

In order to solve these problems, we newly synthesized a conjugate of ursodeoxycholic acid with para-aminobenzoic acid (PABA). In the present paper, a new simple method to estimate outlined condition of the intestinal bacteria using this new compound is going to be discussed.

MATERIALS AND METHODS

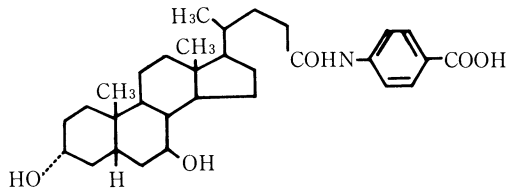
Synthesis of the Conjugate of Ursodeoxycholic Acid with PABA

PABA conjugate of ursodeoxycholic acid was synthesized by modifying the method of Bergström et al[1] who reported a simple method to synthesize glycine or taurine conjugated bile acids.

1.473 g (3.752 m moles) of ursodeoxycholic acid was dissolved in 6 ml of dioxane containing 0.89 ml of tri-n-butylamine. The solution was cooled to +10° and 0.357 ml of ethylchlorocarbonate was added. After 15 min at this temperature a solution of 510 mg of PABA in 3.75 ml of IN sodium hydroxide was added and the

Address for correspondence and reprint requests:
Makoto Takahashi, M.D.,
Department of Surgery, Chugoku Rosai Hospital 1477, Hiro-machi, Kure City, Hiroshima 737-01, Japan.
Telephone: 0823-72-7171, Japan

mixture rapidly mixed. After 15 min, 60 ml of distilled water was added to the reaction mixture. Following acidification with HCl, extraction was carried out with 60 ml of ethyl acetate, three times. The extracts were washed with distilled water and dehydrated with anhydrous sodium sulfate. Then the organic solvent was gradually evaporated to give crystals of the reaction product. Recrystallization was possible from benzene. Thin layer chromatography (TLC) and nuclear magnetic resonance (NMR) demonstrated that this reaction product was definitely PABA-ursodeoxycholic acid conjugate as shown in Fig. 1.



$C_{31}H_{45}NO_6$
mol wt : 527.70

Fig. 1. Chemical structure and molecular weight of PABA-ursodeoxycholic acid

Purity of the compound was examined by high performance thin-layer chromatography (HPTLC) (Kieselgel 60 F_{254} plates, Merck) using as a solvent system of benzene-dioxane-acetic acid (15:5:2, V/V). PABA and its conjugate appeared as apparent spots under short-wave ultraviolet light. Ursodeoxycholic acid and PABA-ursodeoxycholic acid conjugate were further identified by spraying with 5% phosphomolybdate in ethanol. The Rfs of the compounds were: PABA 0.73; ursodeoxycholic acid, 0.68; PABA-ursodeoxycholic acid, 0.49. HPTLC revealed that thus synthesized material was practically pure, having less than 5% contamination with free ursodeoxycholic acid but having no contamination with PABA.

Figure 2 shows thin layer chromatography of PABA-UDCA and related compounds.

In Vitro Hydrolysis of PABA-Ursodeoxycholic Acid with Cholylglycine Hydrolase

It was studied whether PABA was reasonably released or not in incubation experiments with three different amounts of PABA-ursodeoxycholic acid with cholylglycine hydrolase.

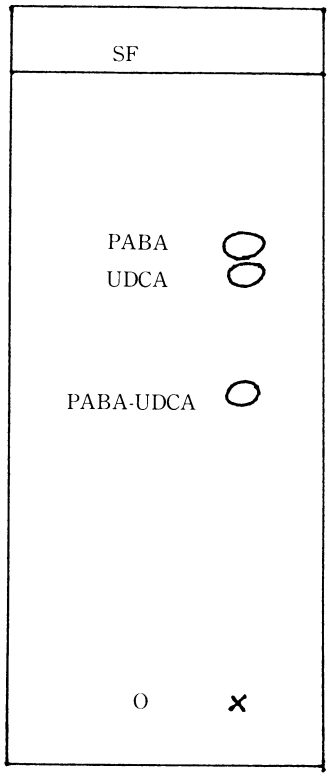


Fig. 2. Thin-layer chromatography of the compounds. Plate: Kieselgel 60 F_{254}; Solvent system: Benzene-dioxane-acetic acid (15:5:2, v/v), SF: Solvent front; PABA: Para-aminobenzoic acid; UDCA: Ursodeoxycholic acid; PABA-UDCA: PABA conjugate of UDCA; O: Origin.

0.4 ml of 0.75% 2-mercaptoethanol and 0.4 ml of 1.86% EDTA solutions were added in 0.4 ml of 0.025 M sodium acetate buffer which was adjusted to pH 5.6. Then 0.6 ml of solutions with three different amounts of substrate in 0.025 M sodium acetate buffer (pH 5.6) were added to each samples: The solutions contained 0.15 mg (Solution A), 1.5 mg (Solution B) and 4.0 mg (Solution C) in 0.6 ml, respectively. Following the addition of 0.2 ml of cholylglycine hydrolase solution (2 mg/ml of water; Sigma Chemical Company, St. Louis, MO, U.S.A.), incubation was started at 37°C in water bath. After incubation, the amount of PABA released was determined by sampling at 1, 5, 10, 20, 30 and 45 min. PABA determination was possible by the measurement of the absorbance at 550 nm in spectrophotometer using an assay system for

urinary PABA in PFD test (Eisai Co., Tokyo, Japan).

Animal Models (*In Vivo* Experiment)

Twenty seven male Sprague-Dawley rats (200—300 g) were divided into three experimental groups as follows.

Control group (7 rats): Each rat of this group was placed in individual stainless metabolic cage, and 6-hr urine collection was made for the measurement of the amount of background PABA. Then, 10 mg of PABA - ursodeoxycholic acid in a slightly basic (pH 7.5) aqueous solution was orally administered to each rat using Mark needle, and 6-hr urine was collected just after dosing. The amounts of PABA in this 6-hr-collected urine samples were determined by the same method as *in vitro* study. Cumulative amount of urinary PABA which was excreted through 6 hr after administration, was calculated by subtraction of the amount of background PABA.

Group treated with antibiotics (9 rats): In this group, large amounts of broadspectrum antibiotics were given orally in order to produce rats with gastrointestinal tract which was relatively free of microflora. Antibiotics were administered orally to each rat in a dose of 25 mg doxycycline, 25 mg ampicillin and 25 mg fradiomycin twice a day for three days. Six-hr urine collection was made during the antibiotic treatment for obtaining the amount of background PABA. Following the final administration of antibiotics, 10 mg of the compound was given orally. Urine collection and calculation of urinary PABA were made in the same way as in control group.

Group with intestinal blind loop (11 rats): Animals were fasted overnight before surgery. Under ether anesthesia blind loop was constructed surgically in order to produce rats with intestinal bacterial overgrowth[9]. After transection of the ileum near the ileal end, the oral stump of the ileum was closed whereas the anal stump of the ileum was anastomosed to the intestine at the point 12 cm. proximal to the transected site in end-to-side approximation (Fig. 3). Only water was given for three days postoperatively, and foods were given thereafter. At the 1st week postoperatively, the rat was placed in metabolic cage and 6-hr urine collection was made for the measurement of background PABA. Then urine was collected through six hours after oral administration of 10 mg of PABA-ursodeoxycholic acid. Cumulative urinary PABA in 6 hr after dosing was assayed in the same manner as in the other groups of rats.

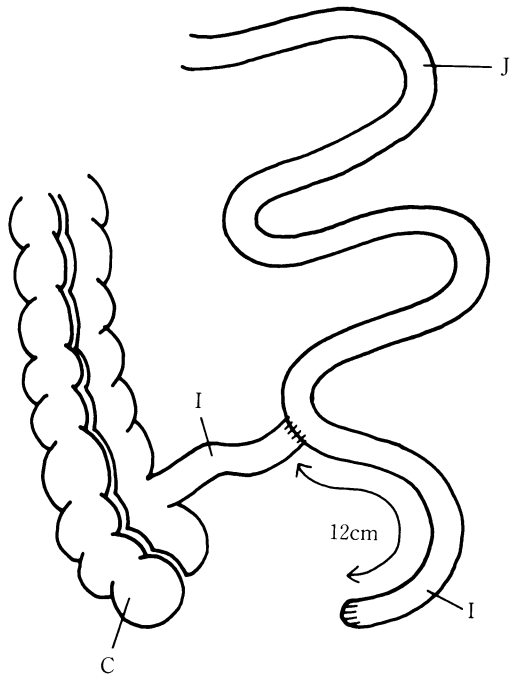


Fig. 3. Sketch of the surgical manipulation for making rats with blind loop. C = Cecum, I = Ileum, J = Jejunum.

RESULTS

***In Vitro* Study**

Figure 4 shows the time course of enzymatic hydrolysis of PABA-ursodeoxycholic acid over 45 min. period at three different concentrations of substrate. This study revealed that this new compound was reasonably hydrolyzed by cholylglycine hydrolase which deconjugates glycine- or taurine- conjugated bile acids.

***In Vivo* Study**

Figure 5 shows the results of the urinary excretion of PABA during 6 hr after dosing for all groups of rats. Control group of rats excreted 140.1 ± 59.5 μg (mean ± SD) of PABA into urine. Significant suppression on urinary PABA excretion was found in antibiotic-treated rats compared with control group [18.3 ± 16.7 μg ($P < 0.001$)]. By contrast, rats with intestinal bacterial overgrowth resulted in greater excretion of PABA with significance than the normals [451.1 ± 223.6 μg ($P < 0.01$)].

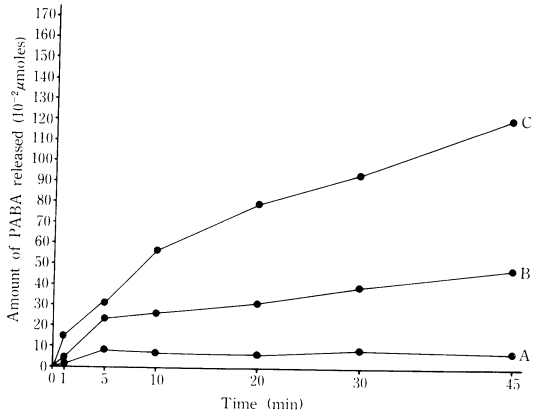


Fig. 4. Time course of enzymatic hydrolysis of PABA-ursodeoxycholic acid by cholylglycine hydrolase in pH 5.6 acetate buffer. Incubation was carried out at three different substrate amounts: A = Solution A, 0.15 mg; B = Solution B, 1.5 mg; C = Solution C, 4.0 mg.

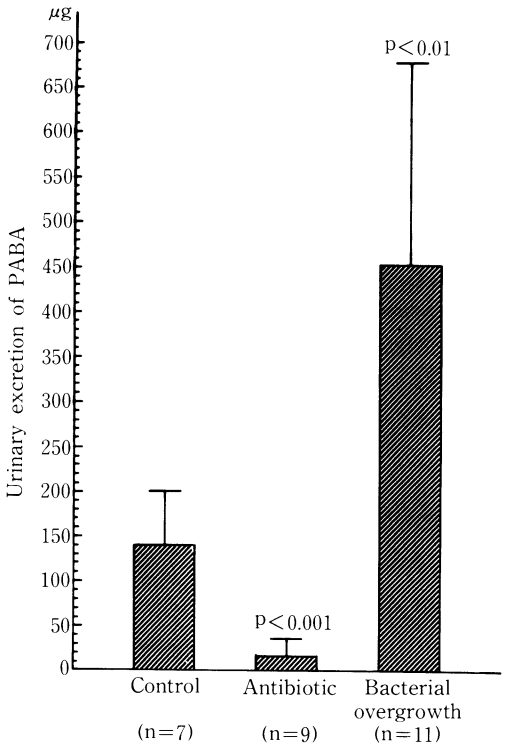


Fig. 5. Cumulative urinary excretion of PABA (mean ± SD) at 6 hr after oral administration of 10 mg PABA-ursodeoxycholic acid to three different groups of rats.

DISCUSSION

In 1972 Imondi et al[6] reported reliable results in animals with a pancreatic function test (PFD test) involving the oral administration of a chymotrypsin-labile peptide which contains para-aminobenzoic acid (PABA) as a tracer group. The principle of this test was based on the distinctive feature of PABA: In the small bowel, in the presence of chymotrypsin, the peptide (N-benzoic-L-tyrosyl-p-aminobenzoic acid; BT-PABA) which is a complex of benzonic acid, tyrosine and PABA, is split resulting in the liberation of PABA, which is rapidly absorbed from the gut, undergoes conjugation in the liver, and is excreted into the urine. Accordingly, the amount of PABA excreted in the urine reflects the activity of chymotrypsin which is secreted from the exocrine glands of the pancreas. In 1976 Gyr et al[4] reported that the procedure using this compound was a new possible oral test of exocrine pancreatic function in human being. Since then, the PFD test has been widely employed for the clinical evaluation of the exocrine pancreatic function.

It is well known that bile acids are synthesized through many steps of enzymatic reactions from cholesterol in the liver, resulting in the production of the primary bile acids such as cholic acid and chenodeoxycholic acid. These primary bile acids are conjugated with glycine or taurine also in the liver, and are excreted into bile as a conjugated form. In the intestine, microorganisms hydrolyze the conjugated bile acids under the action of cholylglycine hydrolase, leading to the production of deconjugated bile acids. Many strains of anaerobic microbes such as *Clostridium*, *Bacteroides*, *Bifidobacterium*, *Eubacterium* have been reported as bacteria capable of bile salt deconjugation.

These facts concerning the distinctive feature of PABA and bile acid metabolism, led us to synthesize a new conjugate of ursodeoxycholic acid with PABA for establishing a new simple diagnostic procedure for the evaluation of intestinal microorganism activities. We selected ursodeoxycholic acid for the synthesis because this bile acid has been widely administered to the patients with cholesterol gallstones without any toxicity.

In our study, *in vitro* analyses indicated that the PABA-ursodeoxycholic acid was reasonably deconjugated by cholylglycine hydrolase which was isolated from *Clostridium perfringens (welchii)*, resulting in the release of PABA. In animal experiment, urinary excretions of PABA were determined during six hr after oral administration of a certain dose of PABA-ursodeoxycholic acid. Treatment with oral administration of massive broadspectrum antibiotics caused rats significant decrease in urinary PABA as a result of remarkable suppression of the intestinal microorganisms ($P < 0.001$). Adversely, urinary excretion of PABA was significantly elevated in rats with blind loop as a result of the intestinal bacterial overgrowth ($P < 0.01$).

There have been several published methods for detecting bacterial overgrowth[2,7,8]-e.g., culture of small-bowel aspirates, analysis of aspirates for products of bacterial activity such as free bile acids, or measurement of bacterial enzymatic activity in bacteria-free filtrates of intestinal content. However, all of these methods can not be accepted as a clinical routine diagnostic procedure because of their inconveniences. In 1971 Fromm and Hofmann[3] reported a test called "breath test" for human use utilizing ^{14}C-labeled glycocholate, which upon bacterial cleavage allows the labeled carbon tracer to be released and metabolized to CO_2. This technique has been shown to be quite reliable for the detection of bacterial overgrowth in humans[10]. However, this breath test has not been widely used for it has serious problems of using radioisotope and being troublesome. The test which we describe in the present paper can solve these problems, and is conceivable to be simple and useful as a clinical diagnostic procedure for the evaluation of intestinal microorganisms. Moreover, this compound might become a weapon for elucidating suitable prescription of the antiseptic agents prior to intestinal surgery, relationship of the intestinal bacteria with colon cancer, and effects of the diet on the intestinal microflora. Wide application of this new compound in various scientific fields would be expected.

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REFERENCES

1. **Bergström, S. and Norman, A.** 1953. Synthesis of conjugated bile acids. Acta Chem. Scand. **7**: 1126—1127.
2. **Egger, G. and Kessler, J.L.** 1973. Clinical experience with a simple test for the detection of bacterial deconjugation of bile salts and the site and extent of bacterial overgrowth in the small intestine. Gastroenterology **64**: 545—551.
3. **Fromm, H. and Hofmann, A.F.** 1971. Breath test for altered bile-acid metabolism. Lancet **2**: 621—625.
4. **Gyr, K., Stalder, G.A. and Schiffmann, I.** 1976. Oral administration of a chymotrypsin-labile peptide-a new test of exocrine pancreatic function in man (PFT). Gut **17**: 27—32.
5. **Hill, M.J.** 1976. Fecal steroids in the etiology of large bowel cancer. *In* P.P. Nair and D. Kritchevsky (ed), The Bile Acids. Volume 3. Plenum Press. 169—200.
6. **Imondi, A.R., Stradley, R.P. and Wohlgemuth, R.L.** 1972. Synthetic peptides in the diagnosis of exocrine pancreatic insufficiency in animals. Gut **13**: 726—731.
7. **Mallory, P., Kern, F., Smith, J. and Savage, D.** 1973. Patterns of bile acids and microflora in the human small intestine. Gastroenterology **64**: 26—33.
8. **O'Moore, R.R.L. and Percy-Robb, I.W.** 1973. Analysis of bile acids and their conjugates in jejunal juice by thin layer chromatography and direct densitometry. Clin. Chim. Acta **43**: 39—47.
9. **Polter, D.E., Boyle, J.D., Miller, L.G. and Finegold, S.M.** 1968. Anaerobic bacteria as cause of the blind loop syndrome. Gastroenterology **54**: 1148—1154.
10. **Sherr, H.P., Sasaki, Y. and Newman, A.** 1971. Detection of bacterial deconjugation of bile salts by a convenient breath-analysis technique. N. Engl. J. Med. **285**: 656—661.